

cause the reversal of particle formation may be, but not limited to, the pH, ionic strength, oxidative or reductive conditions or agents, or enzymatic activity.

### DNA Template Polymerization

5 Low molecular weight cations with valency  $< +3$  fail to condense DNA in aqueous solutions under normal conditions. However, cationic molecules with the charge  $< +3$  can be polymerized in the presence of DNA and the resulting polymers can cause DNA to condense into compact structures. Such an approach is known in synthetic polymer chemistry as template polymerization. During this process, monomers (which are initially weakly associated with the template) are positioned along template's backbone, thereby promoting their polymerization. Weak electrostatic association of the nascent polymer and the template becomes stronger with chain growth of the polymer. Trubetskoy et al used two types of polymerization reactions to achieve DNA condensation: step polymerization and chain polymerization (VS Trubetskoy, VG Budker, LJ Hanson, PM Slattum, JA Wolff, LE Hagstrom. Nucleic Acids Res. 26:4178-4185, 1998) U.S. 08/778,657, U.S. 09/000,692, U.S. 97/24089, U.S. 09/070299, and U.S. 09/464,871. Bis(2-aminoethyl)-1,3-propanediamine (AEPD), a tetraamine with 2.5 positive charges per molecule at pH 8 was polymerized in the presence of plasmid DNA using cleavable disulfide amino-reactive cross-linkers dithiobis (succinimidyl propionate) and dimethyl-3,3'-dithiobispropionimidate. Both reactions yielded DNA/polymer complexes with significant retardation in agarose electrophoresis gels demonstrating significant binding and DNA condensation. Treatment of the polymerized complexes with 100 mM dithiothreitol (DTT) resulted in the pDNA returning to its normal supercoiled position following electrophoresis proving thus cleavage the backbone of the. The template dependent polymerization process was also tested using a 14 mer peptide encoding the nuclear localizing signal (NLS) of SV40 T antigen SEQ ID NO: 1 (CGYGPKKKRKVGGC) as a cationic "macromonomer". Other studies included pegylated comonomer (PEG-AEPD) into the reaction mixture and resulted in "worm"-like structures (as judged by transmission electron microscopy) that have previously been observed with DNA complexes formed from block co-polymers of polylysine and PEG (MA Wolfert, EH Schacht, V Toncheva, K Ulbrich, O Nazarova, LW Seymour Human Gene Ther. 7:2123-2133, 1996). Blessing et al used bithiol derivative of spermine and reaction of thiol-disulfide exchange to promote chain growth. The presence of DNA accelerated the polymerization reaction as measured the rate of disappearance of free thiols in the reaction mixture (T Blessing, JS Remy, JP Behr. J. Am. Chem. Soc. 120:8519-8520, 1998).

"Caging" of polycation-condensed DNA particles.

The stability of DNA nanoassemblies based on DNA condensation is generally low *in vivo* because they easily engage in polyion exchange reactions with strong polyanions. The process of exchange consists of two stages: 1) rapid formation of a triple DNA-polycation-polyanion complex, 2) slow substitution of one same-charge polyion with another. At equilibrium conditions, the whole process eventually results in formation of a new binary complex and an excess of a third polyion. The presence of low molecular weight salt can greatly accelerate such exchange reactions, which often result in complete disassembly of condensed DNA particles. Hence, it is desirable to obtain more colloiddally stable structures where DNA would stay in its condensed form in complex with corresponding polycation independently of environment conditions.

The complete DNA condensation upon neutralization of only 90% of the polymer's phosphates results in the presence of unpaired positive charges in the DNA particles. If the polycation contains such reactive groups, such as primary amines, these unpaired positive charges may be modified. This modification allows practically limitless possibilities of modulating colloidal properties of DNA particles via chemical modifications of the complex. We have demonstrated the utility of such reactions using traditional DNA-poly-L-lysine (DNA/PLL) system reacted with the cleavable cross-linking reagent dimethyl-3,3'-dithiobispropionimidate (DTBP) which reacts with primary amino groups with formation of amidines (VS Trubetskoy, A Loomis, PM Slattum, JE Hagstrom, VG Budker, JA Wolff. Bioconjugate Chem. 10:624-628, 1999) U.S. 08/778,657, U.S. 09/000,692, U.S. 97/24089, U.S. 09/070299, and U.S. 09/464,871. Similar results were achieved with other polycations including poly(allylamine) and histone H1. The use of another bifunctional reagent, glutaraldehyde, has been described for stabilization of DNA complexes with cationic peptide CWK18 (RC Adam, KG Rice. J. Pharm. Sci. 739-746, 1999).

Recharging.

The caging approach described above could lead to more colloiddally stable DNA assemblies. However, this approach may not change the particle surface charge. Caging with bifunctional reagents, which preserve positive charge of amino group, keeps the particle positive. However, negative surface charge would be more desirable for many practical applications, i.e. *in vivo* delivery. The phenomenon of surface recharging is well known in colloid chemistry and is described in great detail for lyophobic/lyophilic systems (for

example, silver halide hydrosols). Addition of polyion to a suspension of latex particles with oppositely-charged surface leads to the permanent absorption of this polyion on the surface and, upon reaching appropriate stoichiometry, changing the surface charge to opposite one. This whole process is salt dependent with flocculation to occur upon reaching the  
 5 neutralization point.

We have demonstrated that similar layering of polyelectrolytes can be achieved on the surface of DNA/polycation particles (VS Trubetskoy, A Loomis, JE Hagstrom, VG Budker, JA Wolff. *Nucleic Acids Res.* 27:3090-3095, 1999). The principal DNA-polycation (DNA/pC) complex used in this study was DNA/PLL (1:3 charge ratio) formed in low salt 25 mM HEPES buffer and recharged with increasing amounts of various polyanions. The DNA  
 10 particles were characterized after addition of a third polyion component to a DNA/polycation complex using a new DNA condensation assay (VS Trubetskoy, PM Slattum, JE Hagstrom, JA Wolff, VG Budker. *Anal. Biochem.* 267:309-313, 1999) and static light scattering. It has been found that certain polyanions such as poly(methacrylic acid) and poly(aspartic acid)  
 15 decondensed DNA in DNA/PLL complexes. Surprisingly, polyanions of lower charge density such as succinylated PLL and poly(glutamic acid), even when added in 20-fold charge excess to condensing polycation (PLL) did not decondense DNA in DNA/PLL (1:3) complexes. Further studies have found that displacement effects are salt-dependent. In addition, polyglutamate but not the relatively weaker polyanion succinylated poly-L-lysine (SPLL)  
 20 displaces DNA at higher sodium chloride concentrations. Measurement of z-potential of DNA/PLL particles during titration with SPLL revealed the change of particle surface charge at approximately the charge equivalency point. Thus, it can be concluded that addition of low charge density polyanion to the cationic DNA/PLL particles results in particle surface charge reversal while maintaining condensed DNA core intact. Finally, DNA/polycation complexes  
 25 can be both recharged and crosslinked or caged U.S. 08/778,657, U.S. 09/000,692, U.S. 97/24089, U.S. 09/070299, and U.S. 09/464,871.

#### The Use of pH-Sensitive Lipids, Amphipathic Compounds, and Liposomes for Drug and Nucleic Acid Delivery

30 After the landmark description of DOTMA (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride) [Felgner, P L, Gadek, T R, Holm, M, et al. *Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. USA.* 1987;84:7413-7417], a plethora of cationic lipids have been synthesized. Basically, all the cationic lipids are amphipathic compounds that contain a hydrophobic domain, a spacer, and

positively-charged amine. The hydrophobic domains are typically hydrocarbon chains such as fatty acids derived from oleic or myristic acid. The hydrocarbon chains are often joined either by ether or ester bonds to a spacer such as glycerol. Quaternary amines often compose the cationic groups. Usually, the cationic lipids are mixed with a fusogenic lipid such as

5 DOPE (dioleoyl phosphatidyl ethanolamine) to form liposomes. The mixtures are mixed in chloroform that is then dried. Water is added to the dried lipid film and unilamellar liposomes form during sonication. Multilamellar cationic liposomes and cationic liposomes/DNA complexes prepared by the reverse-phase evaporation method have also been used for transfection. Cationic liposomes have also been prepared by an ethanol injection technique.

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Several cationic lipids contain a spermine group for binding to DNA. DOSPA, the cationic lipid within the LipofectAMINE formulation (Life Technologies) contains a spermine linked via an amide bond and ethyl group to a trimethyl, quaternary amine [Hawley-Nelson, P, Ciccarone, V and Jessee, J. Lipofectamine reagent: A new, higher efficiency

15 polycationic liposome transfection reagent. *Focus* 1993;15:73-79]. A French group has synthesized a series of cationic lipids such as DOGS (dioctadecylglycinespermine) that contain spermine [Remy, J-S, Sirlin, C, Vierling, P, et al. Gene transfer with a series of lipophilic DNA-binding molecules. *Bioconjugate Chem.* 1994;5:647-654]. DNA has also been transfected by lipophilic polylysines which contain dipalmitoylsuccinylglycerol

20 chemically-bonded to low molecular weight (~3000 MW) polylysine [Zhou, X, Kilbanov, A and Huang, L. Lipophilic polylysines mediate efficient DNA transfection in mammalian cells. *Biochim. Biophys. Acta* 1991;1065:8-14. Zhou, X and Huang, L. DNA transfection mediated by cationic liposomes containing lipopolylysine: Characterization and mechanism of action. *Biochim. Biophys. Acta* 1994;1195-203].

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Other studies have used adjuvants with the cationic liposomes. Transfection efficiency into Cos cells was increased when amphiphilic peptides derived from influenza virus hemagglutinin were added to DOTMA/DOPE liposomes [Kamata, H, Yagisawa, H, Takahashi, S, et al. Amphiphilic peptides enhance the efficiency of liposome-mediated DNA transfection. *Nucleic Acids Res.* 1994;22:536-537]. Cationic lipids have been combined with

30 galactose ligands for targeting to the hepatocyte asialoglycoprotein receptor [Remy, J-S, Kichler, A, Mordvinov, V, et al. Targeted gene transfer into hepatoma cells with lipopolyamine-condensed DNA particles presenting galactose ligands: A stage toward artificial viruses. *Proc. Natl. Acad. Sci. USA* 1995;92:1744-1748]. Thiol-reactive phospholipids have also been incorporated into cationic lipid/pDNA complexes to enable

cellular binding even when the net charge of the complex is not positive [Kichler, A, Remy, J-S, Boussif, O, et al. Efficient gene delivery with neutral complexes of lipospermine and thiol-reactive phospholipids. *Biochem. Biophys. Res. Comm.* 1995;209:444-450]. DNA-dependent template process converted thiol-containing detergent possessing high critical  
 5 micelle concentration into dimeric lipid-like molecule with apparently low water solubility (JP Behr and colleagues).

Cationic liposomes may deliver DNA either directly across the plasma membrane or via the endosome compartment. Regardless of its exact entry point, much of the DNA within cationic liposomes does accumulate in the endosome compartment. Several approaches have  
 10 been investigated to prevent loss of the foreign DNA in the endosomal compartment by protecting it from hydrolytic digestion within the endosomes or enabling its escape from endosomes into the cytoplasm. They include the use of acidotropic (lysomotropic), weak amines such as chloroquine that presumably prevent DNA degradation by inhibiting endosomal acidification [Legendre, J. & Szoka, F. Delivery of plasmid DNA into  
 15 mammalian cell lines using pH-sensitive liposomes: Comparison with cationic liposomes. *Pharmaceut. Res.* **9**, 1235-1242 (1992)]. Viral fusion peptides or whole virus have been included to disrupt endosomes or promote fusion of liposomes with endosomes, and facilitate release of DNA into the cytoplasm [Kamata, H., Yagisawa, H., Takahashi, S. & Hirata, H. Amphiphilic peptides enhance the efficiency of liposome-mediated DNA transfection.  
 20 *Nucleic Acids Res.* **22**, 536-537 (1994). Wagner, E., Curiel, D. & Cotten, M. Delivery of drugs, proteins and genes into cells using transferrin as a ligand for receptor-mediated endocytosis. *Advanced Drug Delivery Reviews* **14**, 113-135 (1994)].

Knowledge of lipid phases and membrane fusion has been used to design potentially more versatile liposomes that exploit the endosomal acidification to promote fusion with  
 25 endosomal membranes. Such an approach is best exemplified by anionic, pH-sensitive liposomes that have been designed to destabilize or fuse with the endosome membrane at acidic pH [Duzgunes, N., Straubinger, R.M., Baldwin, P.A. & Papahadjopoulos, D. *pH-sensitive liposomes*. (eds Wilschub, J. & Hoekstra, D.) p. 713-730 (Marcel Deker INC, 1991)]. All of the anionic, pH-sensitive liposomes have utilized phosphatidylethanolamine  
 30 (PE) bilayers that are stabilized at non-acidic pH by the addition of lipids that contain a carboxylic acid group. Liposomes containing only PE are prone to the inverted hexagonal phase (H<sub>II</sub>). In pH-sensitive, anionic liposomes, the carboxylic acid's negative charge increases the size of the lipid head group at pH greater than the carboxylic acid's pK and

thereby stabilizes the phosphatidylethanolamine bilayer. At acidic pH within endosomes, the uncharged or reduced charge species is unable to stabilize the phosphatidylethanolamine-rich bilayer. Anionic, pH-sensitive liposomes have delivered a variety of membrane-impermeant compounds including DNA. However, the negative charge of these pH-sensitive liposomes prevents them from efficiently taking up DNA and interacting with cells; thus decreasing their utility for transfection. We have described the use of cationic, pH-sensitive liposomes to mediate the efficient transfer of DNA into a variety of cells in culture U.S. 08/530,598, and U.S. 09/020,566.

#### 10 The Use of pH-Sensitive Polymers for Drug and Nucleic Acid Delivery

Polymers that pH-sensitive are have found broad application in the area of drug delivery exploiting various physiological and intracellular pH gradients for the purpose of controlled release of drugs (both low molecular weight and polymeric). pH sensitivity can be broadly defined as any change in polymer's physico-chemical properties over certain range of pH. More narrow definition demands significant changes in the polymer's ability to retain (release) a bioactive substance (drug) in a physiologically tolerated pH range (usually pH 5.5 - 8). pH-sensitivity presumes the presence of ionizable groups in the polymer (polyion). All polyions can be divided into three categories based on their ability to donate or accept protons in aqueous solutions: polyacids, polybases and polyampholytes. Use of pH-sensitive polyacids in drug delivery applications usually relies on their ability to become soluble with the pH increase (acid/salt conversion), to form complex with other polymers over change of pH or undergo significant change in hydrophobicity/hydrophilicity balance. Combinations of all three above factors are also possible.

Copolymers of polymethacrylic acid (Eudragit S, Rohm America) are known as polymers which are insoluble at lower pH but readily solubilized at higher pH, so they are used as enteric coatings designed to dissolve at higher intestinal pH (Z Hu et al. J. Drug Target., 7, 223, 1999). A typical example of pH-dependent complexation is copolymers of polyacrylate(graft)ethyleneglycol which can be formulated into various pH-sensitive hydrogels which exhibit pH-dependent swelling and drug release (F Madsen et al., Biomaterials, 20, 1701, 1999). Hydrophobically-modified N-isopropylacrylamide-methacrylic acid copolymer can render regular egg PC liposomes pH-sensitive by pH-dependent interaction of grafted aliphatic chains with lipid bilayer (O Meyer et al., FEBS Lett., 421, 61, 1998). Polymers with pH-mediated hydrophobicity (like polyethylacrylic acid)

can be used as endosomal disruptors for cytoplasmic drug delivery (Murthy, N., Robichaud, J.R., Tirrell, D.A., Stayton, P.S., Hoffman, A.S. J. Controlled Release 61, 137, 1999).

Polybases have found broad applications as agents for nucleic acid delivery in transfection/gene therapy applications due to the fact they readily interact with polyacids.

- 5 A typical example is polyethylenimine (PEI). This polymer secures nucleic acid electrostatic adsorption on the cell surface followed by endocytosis of the whole complex. Cytoplasmic release of the nucleic acid occurs presumably via the so called "proton sponge" effect according to which pH-sensitivity of PEI is responsible for endosome rupture due to osmotic swelling during its acidification (O Boussif et al. Proc. Natl. Acad. Sci. USA 92, 7297, 1995).
- 10 Cationic acrylates possess the similar activity (for example, poly-((2-dimethylamino)ethyl methacrylate) (P van de Wetering et al. J. Controlled Release 64, 193, 2000). However, polybases due to their polycationic nature pH-sensitive polybases have not found broad *in vivo* application so far, due to their acute systemic toxicity *in vivo* (JH Senior, Biochim. Biophys. Acta, 1070, 173, 1991). Milder polybases (for example, linear PEI) are better
- 15 tolerated and can be used systemically for *in vivo* gene transfer (D Goula et al. Gene Therapy 5, 712, 1998).

#### Endosome Disruption

- Many biologically active compounds, in particular large and/or charged compounds, are
- 20 incapable of crossing biological membranes. In order for these compounds to enter cells they must either be taken up by the cells via endocytosis, into endosomes, or there must be a disruption of the cellular membrane to allow the compound to cross. In the case of endosomal entry, the endosomal membrane must be disrupted to allow for the entrance of the compound in the interior of the cell. Therefore, either entry pathway into the cell requires a
- 25 disruption of the cellular membrane. There exist compounds termed membrane active compounds that disrupt membranes. One can imagine that if the membrane active agent were operative in a certain time and place it would facilitate the transport of the biologically active compound across the biological membrane. The control of when and where the membrane active compound is active is crucial to effective transport. If the membrane active compound
- 30 is too active or active at the wrong time, then no transport occurs or transport is associated with cell rupture and thereby cell death. Nature has evolved various strategies to allow for membrane transport of biologically active compounds including membrane fusion and the use membrane active compounds whose activity is modulated such that activity assists transport without toxicity. Many lipid-based transport formulations rely on membrane fusion

and some membrane active peptides' activities are modulated by pH. In particular, viral coat proteins are often pH-sensitive, inactive at neutral or basic pH and active under the acidic conditions found in the endosome.

## 5 Small Molecular Endosomolytic Agents

A cellular transport step that has attracted attention for gene transfer is that of DNA release from intracellular compartments such as endosomes (early and late), lysosomes, phagosomes, vesicle, endoplasmic reticulum, golgi apparatus, trans golgi network (TGN), and sarcoplasmic reticulum. Release includes movement out of an intracellular compartment  
 10 into cytoplasm or into an organelle such as the nucleus. A number of chemicals such as chloroquine, bafilomycin or Brefeldin A1 have been used to disrupt or modify the trafficking of molecules through intracellular pathways. Chloroquine decreases the acidification of the endosomal and lysosomal compartments but also affects other cellular functions. Brefeldin A, an isoprenoid fungal metabolite, collapses reversibly the Golgi apparatus into the  
 15 endoplasmic reticulum and the early endosomal compartment into the trans-Golgi network (TGN) to form tubules. Bafilomycin A<sub>1</sub>, a macrolide antibiotic is a more specific inhibitor of endosomal acidification and vacuolar type H<sup>+</sup>-ATPase than chloroquine.

## Viruses, Proteins and Peptides for Disruption of Endosomes and Endosomal Function

20 Viruses such as adenovirus have been used to induce gene release from endosomes or other intracellular compartments (D. Curiel, Agarwal, S., Wagner, E., and Cotten, M. PNAS 88:8850, 1991). Rhinovirus has also been used for this purpose (W. Zauner et al. J. Virology 69:1085-92, 1995). Viral components such as influenza virus hemagglutinin subunit HA-2 analogs has also been used to induce endosomal release (E. Wagner et al. PNAS 89:7934,  
 25 1992). Amphipathic peptides resembling the N-terminal HA-2 sequence has been studied (K. Mechtler and E. Wagner, New J. Chem. 21:105-111, 1997). Parts of the pseudomonas exotoxin and diphtheria toxin have also been used for drug delivery (I. Pastan and D. FitzGerald. J. Biol. Chem. 264:15157, 1989).

A variety of synthetic amphipathic peptides have been used to enhance transfection of  
 30 genes (N. Ohmori et al. Biochem. Biophys. Res. Commun. 235:726, 1997). The ER-retaining signal (KDEL sequence) has been proposed to enhance delivery to the endoplasmic reticulum and prevent delivery to lysosomes (S. Seetharam et al. J. Biol. Chem. 266:17376, 1991).

Other Cellular and Intracellular Gradients Useful for Delivery

Nucleic acid and gene delivery may involve the biological pH gradient that is active within organisms as a factor in delivering a polynucleotide to a cell. Different pathways that may be affected by the pH gradient include cellular transport mechanisms, endosomal disruption/breakdown, and particle disassembly (release of the DNA). Other gradients that can be useful in gene therapy research involve ionic gradients that are related to cells. For example, both  $\text{Na}^+$  and  $\text{K}^+$  have large concentration gradients that exist across the cell membrane. Systems containing metal-binding groups can utilize such gradients to influence delivery of a polynucleotide to a cell. Changes in the osmotic pressure in the endosome also have been used to disrupt membranes and allow for transport across membrane layer. Buffering of the endosome pH may cause these changes in osmotic pressure. For example, the "proton sponge" effect of PEI (O Boussif et al. Proc. Natl. Acad. Sci. USA 92, 7297, 1995) and certain polyanions (Murthy, N., Robichaud, J.R., Tirrell, D.A., Stayton, P.S., Hoffman, A.S. Journal of Controlled Release 1999, 61, 137) are postulated to cause an increase in the ionic strength inside of the endosome, which causes a increase in osmotic pressure. This pressure increase results in membrane disruption and release of the contents of the endosome.

In addition to pH and other ionic gradients, there exist other difference in the chemical environment associated with cellular activities that may be used in gene delivery. In particular enzymatic activity both extra and intracellularly may be used to deliver the gene of interest either by aiding in the delivery to the cell or escape from intracellular compartments. Proteases, found in serum, lysosome and cytoplasm, may be used to disrupt the particle and allow its interaction with the cell surface or cause it fracture the intracellular compartment, *e.g.* endosome or lysosome, allowing the gene to be released intracellularly.

**SUMMARY OF THE INVENTION**

The invention relates to noncovalent amphiphile binding systems for use in biologic systems. More particularly, amphiphile-binding agents and polymers of amphiphile-binding agents are utilized in the delivery of molecules, polymers, nucleic acids and genes to cells.

Described in a preferred embodiment is a process for obtaining an expression product by delivering a polynucleotide to a cell, comprising the step of associating an amphiphile

binding agent, an amphiphile, and a polynucleotide to form a complex. Then, delivering the complex to the cell and expressing the polynucleotide in the cell.

In another preferred embodiment, a complex is described for delivering and expressing DNA  
5 in a mammal, comprising an amphiphile binding agent, an amphiphile, and DNA in complex.

Another preferred embodiment is a process for obtaining an expression product *in vivo*, comprising forming a complex with a cyclodextrin, an amphiphile and a polynucleotide. Then, delivering the complex to a cell in a mammal which expresses the polynucleotide.

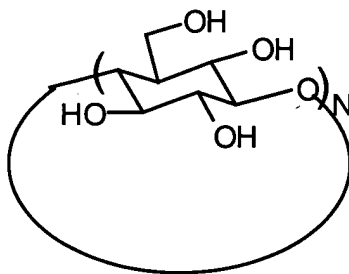
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### DETAILED DESCRIPTION OF THE INVENTION

The following description provides exemplary embodiments of the systems, compositions, and methods of the present invention. These embodiments include a variety of  
15 systems that have been demonstrated as effective delivery systems. The invention is not limited to these particular embodiments.

#### Cyclodextrin structure and binding properties

20 Cyclodextrins are naturally occurring cyclic oligomers of glucose in 1-4  $\alpha$  linkages (structure 1).



25 Cyclodextrin composed of six glucose units ( $N=6$ ) is called  $\alpha$ -cyclodextrin, 7 units is called  $\beta$ -cyclodextrin, and 8 is called  $\gamma$ -cyclodextrin. The cyclic structure is torroidal in shape with the center of the torroid relatively nonpolar compared to water. For this reason, cyclodextrins will bind to nonpolar sections of amphipathic compounds, also known as amphiphilic

compounds or amphiphiles, in water. Amphiphiles are compounds that contain both hydrophilic and hydrophobic functional groups. Examples include lipids, acyl-glycerol, sterols, polyethyleneglycol, and amino acids. Hydrophilic groups indicate in qualitative terms that the chemical moiety is water-preferring. Typically, such chemical groups are water soluble, and are hydrogen bond donors or acceptors with water. Examples of hydrophilic groups include compounds with the following chemical moieties; carbohydrates, polyoxyethylene, peptides, oligonucleotides and groups containing amines, amides, alkoxy amides, carboxylic acids, sulfurs, or hydroxyls. Hydrophobic groups indicate in qualitative terms that the chemical moiety is water-avoiding. Typically, such chemical groups are not water soluble, and tend not to hydrogen bonds. Hydrocarbons are hydrophobic groups. Amphipathic compounds bound by cyclodextrins include hydrophobic amino acids (e.g. leucine and phenylalanine), surfactants (e.g. sodium dodecylsulfate and Triton X-100), and lipids (e.g. palmitic acid). The strength of the interaction between cyclodextrin and an amphiphilic compound depends on the size of both the hydrophobic part of the amphiphile and the cyclodextrin. For example,  $\alpha$ -cyclodextrin will bind linear alkyl chains, but not branched tertiary alkyl groups, which are bound by  $\beta$ -cyclodextrin (Stella, V.J., Rajewski, R.A. *Pharm. Res.* 1997, 14, 556. Stella, V.J., Rao, V.M., Zannov, E.A., Zia, V. *Adv. Drug Del. Rev.* 1999, 36, 3.).

#### 20 Nucleic Acid delivery by polycations and cationic lipids

There are many nonviral nucleic acid complexes that have been shown to aid in delivery of DNA into cells. Nucleic acid includes DNA (plasmid DNA, antisense, oligonucleotides) and RNA (ribozymes, oligonucleotides, artificial messenger RNA). In general, these nonviral complexes may be grouped into two classes: cationic lipid complexes (lipoplexes) and cationic polymer (polyplexes) complexes. In either case, the polyanionic DNA is complexed with a cation. In lipoplexes, the cations are associated noncovalently by hydrophobic lipid-lipid interactions to form a polycation. In polymer complexes, the positive charges are attached covalently to form a polycation. Nucleic acids are delivered to cells for the purpose of gene therapy and antisense therapy.

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#### Nucleic Acids Complexes Containing Cyclodextrins

As mentioned previously, cyclodextrins form complexes with amphipathic molecules that may be positively or negatively charged. Therefore, a polymer composed of

cyclodextrins will become a polyion, a noncovalent amphiphilic electrolyte, when associated with a charged amphiphile. For example, association between a polymer composed of cyclodextrins and a cationic amphiphile will result in a polycation that may interact with DNA. In a preferred embodiment, a cyclodextrin-containing polymers are constructed by  
 5 reacting cyclodextrin with epichlorohydrin under alkaline conditions to produce cyclodextrin-epichlorohydrin copolymer. This cyclodextrin-epichlorohydrin copolymer, compacts pDNA upon addition of cations such as 1-adamantanamine or 1-dodecylamine. The complex of cyclodextrin-epichlorohydrin copolymer and 1-adamantanamine or 1-dodecylamine is a cationic noncovalent amphiphilic polyelectrolyte, which is capable of condensing DNA. In  
 10 addition, cationic amphiphiles that are polymers that are bound to monomeric or polymeric amphiphile binding agents may be used to compact DNA. Such DNA-containing complexes may be used for transfection of cells.

Amphiphile binding agents may also be used to create anionic noncovalent  
 15 amphiphilic polyelectrolytes. Association between a polymer composed of cyclodextrins and an anionic amphiphile will result in a polyanion that will interact with a positively-charged DNA-polycation complex, i.e. "recharge" the DNA complex. In a preferred embodiment, the complex between cyclodextrin-epichlorohydrin copolymer and 4-t-butylbenzoic acid, to form an anionic noncovalent amphiphilic polyelectrolyte, was added to particles of DNA and  
 20 poly-L-lysine. The resulting particles were found to transfect cells in vitro. In addition, anionic amphiphiles that are polymers that are bound to monomeric or polymeric amphiphile binding agents may be used to "recharge" DNA particles. For example, succinylleoyl-poly-L-lysine is an anionic polymeric amphiphile which complexes with the amphiphile binding agent  $\beta$ -cyclodextrin and interacts ("recharges") a poly-L-lysine condensed DNA particle.  
 25 The addition of the cyclodextrin increased the transfection of the recharged particle 33 fold over recharged particle in the absence of cyclodextrin.

Not only is the cyclodextrin the basis for the DNA-polyion interaction, but cyclodextrin-based polyions may have properties (e.g. surface charge and stability) different from standard polyions. In contrast to standard polyions, the polyions derived from  
 30 cyclodextrin-containing polymers and charged amphiphiles are reversible. The existence of the polyion is dependent upon the concentration of the cyclodextrin-containing polymer and the charged amphiphile, such that the disruption of the polyion maybe trigger by simple dilution of either cyclodextrin or charged amphiphile.

Monomeric cyclodextrins may also be incorporated into nucleic acid complexes by association with amphiphile molecules in a DNA complex. In this case, the cyclodextrins are not the basis for the DNA-electrolyte interactions, but may be used to change the properties of the DNA-electrolyte complex, e.g. stability or surface charge. The addition of

5 cyclodextrin into a DNA particle also adds hydrophilic, but not charged, moieties to the particle. Hydrophilic molecules (e.g. PEG) have been shown to increase solubility of DNA particles, decrease the surface charge and thereby increase their stability. Cyclodextrins have the ability to bind to other nonionic hydrophilic molecules such as PEG. Therefore, addition of PEG to a cyclodextrin-containing DNA particle will result in PEG-particle interactions,

10 which may confer the particle with added stability. Unlike other examples of PEG stabilization of DNA particles, the interaction between DNA particle and PEG is transient and may release under dilute, delivery conditions. The rate at which the PEG may be released may be altered by the number of PEG molecules incorporated, the number of cyclodextrins, and the incorporation of PEG derivatives with strong cyclodextrin binding

15 regions (e.g. *t*-octylphenyl group of Triton X-100). In a preferred embodiment, addition of the PEG-derived detergent Triton X-100 to particles of DNA and poly-L-lysine-succinyl- $\beta$ -cyclodextrin resulted in particles that were more stable than particles without addition of the Triton X-100.

Likewise, cell targeting ligands aid in transport to a cell but may not be necessary, and

20 may inhibit, transport into a cell. In all of these cases, the reversible attachment of the interaction modifier, through a labile bond, would be beneficial.

The present invention provides for the transfer of polynucleotides, and other biologically active compounds into cells in culture (also known as “*in vitro*”). Compounds or

25 kits for the transfection of cells in culture is commonly sold as “transfection reagents” or “transfection kits”. The present invention also provides for the transfer of polynucleotides, and biologically active compounds into cells within tissues *in situ* and *in vivo*, and delivered intravascularly (U.S. patent application serial number 08/571,536), intrarterially, intravenous, orally, intraduodenally, via the jejunum (or ileum or colon), rectally, transdermally,

30 subcutaneously, intramuscularly, intraperitoneally, intraparenterally, via direct injections into tissues such as the liver, lung, heart, muscle, spleen, pancreas, brain (including intraventricular), spinal cord, ganglion, lymph nodes, lymphatic system, adipose tissues, thyroid tissue, adrenal glands, kidneys, prostate, blood cells, bone marrow cells, cancer cells, tumors, eye retina, via the bile duct, or via mucosal membranes such as in the mouth, nose,

throat, vagina or rectum or into ducts of the salivary or other exocrine glands. Compounds for the transfection of cells *in vivo* in a whole organism can be sold as “*in vivo* transfection reagents” or “*in vivo* transfection kits” or as a pharmaceutical for gene therapy.

## 5 Definitions

To facilitate an understanding of the present invention, a number of terms and phrases are defined below:

### Amphiphile Binding Agent

10 Amphiphile binding agents are compounds with molecular weight 1,300 or less that bind through a noncovalent interaction amphiphilic compounds in water. The basis for this interaction is contact between hydrophobic portions of the amphiphile with hydrophobic portions of the amphiphile binding agent. In particular  $\alpha$ ,  $\beta$  and  $\gamma$ -cyclodextrins, and their derivatives, are amphiphile binding agents.

15

### Polymeric Amphiphile Binding Agent

Polymeric amphiphile binding agent is a polymer composed of monomers that are amphiphile binding agents.

## 20 Noncovalent Amphiphilic Electrolytes

Noncovalent amphiphilic polyelectrolytes are systems composed of amphiphile binding agents and charged amphiphiles, which are bound by the amphiphile binding agents. The interaction between charged amphiphile and polymer results in a complex that has a different charge than the amphiphile binding agent alone. The amphiphile binding agent may  
25 be uncharged, charge positive or neutral, but upon interaction with a charged amphiphile the charge of the complex is different than the amphiphile binding agent alone.

### Biologically active compound

A biologically active compound is a compound having the potential to react with  
30 biological components. More particularly, biologically active compounds utilized in this specification are designed to change the natural processes associated with a living cell. For purposes of this specification, a cellular natural process is a process that is associated with a cell before delivery of a biologically active compound. In this specification, the cellular production of, or inhibition of a material, such as a protein, caused by a human assisting a

molecule to an *in vivo* cell is an example of a delivered biologically active compound. Pharmaceuticals, proteins, peptides, polypeptides, enzyme inhibitors, hormones, cytokines, antigens, viruses, oligonucleotides, enzymes and nucleic acids are examples of biologically active compounds.

5

### **Peptide and Polypeptide**

Peptide and polypeptide refer to a series of amino acid residues, more than two, connected to one another by amide bonds between the beta or alpha-amino group and carboxyl group of contiguous amino acid residues. The amino acids may be naturally occurring or synthetic. Polypeptide includes proteins and peptides, modified proteins and peptides, and non-natural proteins and peptides. Enzymes are proteins evolved by the cells of living organisms for the specific function of catalyzing chemical reactions. A chemical reaction is defined as the formation or cleavage of covalent or ionic bonds. Bioactive compounds may be used interchangeably with biologically active compound for purposes of this application.

15

### **Cyclodextrin**

A cyclic oligomer of alpha-D-glucopyranose.

### **Delivery of Biologically active compound**

20

The delivery of a biologically active compound is commonly known as "drug delivery". "Delivered" means that the biologically active compound becomes associated with the cell or organism. The compound can be in the circulatory system, intravessel, extracellular, on the membrane of the cell or inside the cytoplasm, nucleus, or other organelle of the cell.

25

Parenteral routes of administration include intravascular (intravenous, intraarterial), intramuscular, intraparenchymal, intradermal, subdermal, subcutaneous, intratumor, intraperitoneal, intrathecal, subdural, epidural, and intralymphatic injections that use a syringe and a needle or catheter. An intravascular route of administration enables a polymer or polynucleotide to be delivered to cells more evenly distributed and more efficiently expressed than direct injections. Intravascular herein means within a tubular structure called a vessel that is connected to a tissue or organ within the body. Within the cavity of the tubular structure, a bodily fluid flows to or from the body part. Examples of bodily fluid include blood, cerebrospinal fluid (CSF), lymphatic fluid, or bile. Examples of vessels include

30

arteries, arterioles, capillaries, venules, sinusoids, veins, lymphatics, and bile ducts. The intravascular route includes delivery through the blood vessels such as an artery or a vein. An administration route involving the mucosal membranes is meant to include nasal, bronchial, inhalation into the lungs, or via the eyes. Other routes of administration include

5 intraparenchymal into tissues such as muscle (intramuscular), liver, brain, and kidney.

Transdermal routes of administration have been effected by patches and iontophoresis.

Other epithelial routes include oral, nasal, respiratory, and vaginal routes of administration.

### **Delivery System**

Delivery system is the means by which a biologically active compound becomes  
10 delivered. That is all compounds, including the biologically active compound itself, that are required for delivery and all procedures required for delivery including the form (such volume and phase (solid, liquid, or gas)) and method of administration (such as but not limited to oral or subcutaneous methods of delivery).

### **15 Nucleic Acid**

The term "nucleic acid" is a term of art that refers to a polymer containing at least two nucleotides. "Nucleotides" contain a sugar deoxyribose (DNA) or ribose (RNA), a base, and a phosphate group. Nucleotides are linked together through the phosphate groups. "Bases" include purines and pyrimidines, which further include natural compounds adenine, thymine,  
20 guanine, cytosine, uracil, inosine, and natural analogs, and synthetic derivatives of purines and pyrimidines, which include, but are not limited to, modifications which place new reactive groups such as, but not limited to, amines, alcohols, thiols, carboxylates, and alkylhalides. Nucleotides are the monomeric units of nucleic acid polymers. A "polynucleotide" is distinguished here from an "oligonucleotide" by containing more than 80  
25 monomeric units; oligonucleotides contain from 2 to 80 nucleotides. The term nucleic acid includes deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). The term encompasses sequences that include any of the known base analogs of DNA and RNA including, but not limited to, 4-acetylcytosine, 8-hydroxy-N6-methyladenosine, aziridinylcytosine, pseudoisocytosine, 5-(carboxyhydroxymethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-  
30 carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, dihydrouracil, inosine, N6-isopentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine,

5'-methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N<sup>6</sup>-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, oxybutoxosine, pseudouracil, queosine, 2-thiocyto-sine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocyto-sine, and 2,6-diaminopurine.

DNA may be in the form of anti-sense, plasmid DNA, parts of a plasmid DNA, product of a polymerase chain reaction (PCR), vectors (P1, PAC, BAC, YAC, artificial chromosomes), expression cassettes, chimeric sequences, chromosomal DNA, or derivatives of these groups. RNA may be in the form of oligonucleotide RNA, tRNA (transfer RNA), snRNA (small nuclear RNA), rRNA (ribosomal RNA), mRNA (messenger RNA), anti-sense RNA, ribozymes, chimeric sequences, or derivatives of these groups.

"Anti-sense" is a polynucleotide that interferes with the function of DNA and/or RNA. This may result in suppression of expression. Natural nucleic acids have a phosphate backbone, artificial nucleic acids may contain other types of backbones and bases. These include PNAs (peptide nucleic acids), phosphothionates, and other variants of the phosphate backbone of native nucleic acids. In addition, DNA and RNA may be single, double, triple, or quadruple stranded.

The term "recombinant DNA molecule" as used herein refers to a DNA molecule that is comprised of segments of DNA joined together by means of molecular biological techniques. "Expression cassette" refers to a natural or recombinantly produced polynucleotide molecule that is capable of expressing protein(s). A DNA expression cassette typically includes a promoter (allowing transcription initiation), and a sequence encoding one or more proteins. Optionally, the expression cassette may include transcriptional enhancers, non-coding sequences, splicing signals, transcription termination signals, and polyadenylation signals. An RNA expression cassette typically includes a translation initiation codon (allowing translation initiation), and a sequence encoding one or more proteins. Optionally, the expression cassette may include translation termination signals, a polyadenosine sequence, internal ribosome entry sites (IRES), and non-coding sequences.

A nucleic acid can be used to modify the genomic or extrachromosomal DNA sequences. This can be achieved by delivering a nucleic acid that is expressed. Alternatively, the nucleic acid can effect a change in the DNA or RNA sequence of the target cell. This can be achieved by homologous recombination, gene conversion, or other, yet to be described, mechanisms.

## Gene

The term "gene" refers to a nucleic acid (*e.g.*, DNA) sequence that comprises coding sequences necessary for the production of a polypeptide or precursor (*e.g.*, -myosin heavy chain). The polypeptide can be encoded by a full length coding sequence or by any portion of the coding sequence so long as the desired activity or functional properties (*e.g.*, enzymatic activity, ligand binding, signal transduction, etc.) of the full-length or fragment are retained. The term also encompasses the coding region of a structural gene and the including sequences located adjacent to the coding region on both the 5' and 3' ends for a distance of about 1 kb or more on either end such that the gene corresponds to the length of the full-length mRNA. The sequences that are located 5' of the coding region and which are present on the mRNA are referred to as 5' non-translated sequences. The sequences that are located 3' or downstream of the coding region and which are present on the mRNA are referred to as 3' non-translated sequences. The term "gene" encompasses both cDNA and genomic forms of a gene. A genomic form or clone of a gene contains the coding region interrupted with non-coding sequences termed "introns" or "intervening regions" or "intervening sequences." Introns are segments of a gene which are transcribed into nuclear RNA (hnRNA); introns may contain regulatory elements such as enhancers. Introns are removed or "spliced out" from the nuclear or primary transcript; introns therefore are absent in the messenger RNA (mRNA) transcript. The mRNA functions during translation to specify the sequence or order of amino acids in a nascent polypeptide.

As used herein, the terms "nucleic acid molecule encoding," "DNA sequence encoding," and "DNA encoding" refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along the polypeptide (protein) chain. The DNA sequence thus codes for the amino acid sequence.

As used herein, the terms "an oligonucleotide having a nucleotide sequence encoding a gene" and "polynucleotide having a nucleotide sequence encoding a gene," means a nucleic acid sequence comprising the coding region of a gene or in other words the nucleic acid sequence which encodes a gene product. The coding region may be present in either a cDNA, genomic DNA or RNA form. When present in a DNA form, the oligonucleotide or polynucleotide may be single-stranded (*i.e.*, the sense strand) or double-stranded. Suitable control elements such as enhancers/promoters, splice junctions, polyadenylation signals, *etc.* may be placed in close proximity to the coding region of the gene if needed to permit proper initiation of transcription and/or correct processing of the primary RNA transcript.

Alternatively, the coding region utilized in the expression vectors of the present invention may contain endogenous enhancers/promoters, splice junctions, intervening sequences, polyadenylation signals, *etc.* or a combination of both endogenous and exogenous control elements.

5           The term "isolated" when used in relation to a nucleic acid, as in "an isolated oligonucleotide" or "isolated polynucleotide" refers to a nucleic acid sequence that is identified and separated from at least one contaminant nucleic acid with which it is ordinarily associated in its natural source. Isolated nucleic acid is such present in a form or setting that is different from that in which it is found in nature. In contrast, non-isolated nucleic acids as  
10   nucleic acids such as DNA and RNA found in the state they exist in nature. For example, a given DNA sequence (*e.g.*, a gene) is found on the host cell chromosome in proximity to neighboring genes; RNA sequences, such as a specific mRNA sequence encoding a specific protein, are found in the cell as a mixture with numerous other mRNAs that encode a multitude of proteins. However, isolated nucleic acid encoding a given protein includes, by  
15   way of example, such nucleic acid in cells ordinarily expressing the given protein where the nucleic acid is in a chromosomal location different from that of natural cells, or is otherwise flanked by a different nucleic acid sequence than that found in nature. The isolated nucleic acid, oligonucleotide, or polynucleotide may be present in single-stranded or double-stranded form. When an isolated nucleic acid, oligonucleotide or polynucleotide is to be utilized to  
20   express a protein, the oligonucleotide or polynucleotide will contain at a minimum the sense or coding strand (*i.e.*, the oligonucleotide or polynucleotide may be single-stranded), but may contain both the sense and anti-sense strands (*i.e.*, the oligonucleotide or polynucleotide may be double-stranded).

## 25   **Gene Expression**

As used herein, the term "gene expression" refers to the process of converting genetic information encoded in a gene into RNA (*e.g.*, mRNA, rRNA, tRNA, or snRNA) through "transcription" of the gene (*i.e.*, via the enzymatic action of an RNA polymerase), and for protein encoding genes, into protein through "translation" of mRNA. Gene expression can be  
30   regulated at many stages in the process. "Up-regulation" or "activation" refers to regulation that increases the production of gene expression products (*i.e.*, RNA or protein), while "down-regulation" or "repression" refers to regulation that decrease production. Molecules (*e.g.*, transcription factors) that are involved in up-regulation or down-regulation are often called "activators" and "repressors," respectively.

**Delivery of Nucleic Acids**

The process of delivering a polynucleotide to a cell has been commonly termed "transfection" or the process of "transfecting" and also it has been termed "transformation".

- 5 The polynucleotide could be used to produce a change in a cell that can be therapeutic. The delivery of polynucleotides or genetic material for therapeutic and research purposes is commonly called "gene therapy". The delivery of nucleic acid can lead to modification of the DNA sequence of the target cell.

- 10 The polynucleotides or genetic material being delivered are generally mixed with transfection reagents prior to delivery. The term "transfection" as used herein refers to the introduction of foreign DNA into eukaryotic cells. Transfection may be accomplished by a variety of means known to the art including calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, retroviral infection, and
- 15 biolistics.

The term "stable transfection" or "stably transfected" refers to the introduction and integration of foreign DNA into the genome of the transfected cell. The term "stable transfectant" refers to a cell which has stably integrated foreign DNA into the genomic DNA.

- 20 The term "transient transfection" or "transiently transfected" refers to the introduction of foreign DNA into a cell where the foreign DNA fails to integrate into the genome of the transfected cell. The foreign DNA persists in the nucleus of the transfected cell for several days. During this time the foreign DNA is subject to the regulatory controls that govern the expression of endogenous genes in the chromosomes. The term "transient transfectant" refers to cells which have taken up foreign DNA but have failed to integrate this DNA. The term
- 25 "naked polynucleotides" indicates that the polynucleotides are not associated with a transfection reagent or other delivery vehicle that is required for the polynucleotide to be delivered to a cell.

- A "transfection reagent" or "delivery vehicle" is a compound or compounds that bind(s) to or complex(es) with oligonucleotides, polynucleotides, or other desired compounds
- 30 and mediates their entry into cells. Examples of transfection reagents include, but are not limited to, cationic liposomes and lipids, polyamines, calcium phosphate precipitates, histone proteins, polyethylenimine, and polylysine complexes (polyethylenimine and polylysine are both toxic). Typically, when used for the delivery of nucleic acids, the transfection reagent has a net positive charge that binds to the polynucleotide's negative charge. For example,

cationic liposomes or polylysine complexes have net positive charges that enable them to bind to DNA or RNA.

### **Enzyme**

5            Enzyme is a protein that acts as a catalyst. That is a protein that increases the rate of a chemical reaction without itself undergoing any permanent chemical change. The chemical reactions that are catalyzed by an enzyme are termed enzymatic reactions and chemical reactions that are not are termed nonenzymatic reactions.

### **10    Complex**

Two molecules are combined, to form a complex through a process called complexation or complex formation, if they are in contact with one another through noncovalent interactions such as electrostatic interactions, hydrogen bonding interactions, and hydrophobic interactions.

15

### **Modification**

A molecule is modified, to form a modification through a process called modification, by a second molecule if the two become bonded through a covalent bond. That is, the two molecules form a covalent bond between an atom from one molecule and an atom from the  
20    second molecule resulting in the formation of a new single molecule. A chemical covalent bond is an interaction, bond, between two atoms in which there is a sharing of electron density.

### **Osmosis**

25            Osmosis is the passage of a solvent through a semipermeable membrane, a membrane through which solvent can pass but not all solutes, separating two solutions of different concentrations. There is a tendency for the separated solutions to become the same concentration as the solvent passes from low concentration to high concentration. Osmosis will stop when the two solutions become equal in concentration or when pressure is applied  
30    to the solution containing higher concentration. When the higher concentrated solution is in a closed system, that is when system is of constant volume, there is a build up of pressure as the solvent passes from low to high concentration. This build up of pressure is called osmotic pressure.

**Salt**

A salt is any compound containing ionic bonds, that is bonds in which one or more electrons are transferred completely from one atom to another.

5 **Interpolyelectrolyte Complexes**

An interpolyelectrolyte complex is a noncovalent interaction between polyelectrolytes of opposite charge.

**Charge, Polarity, and Sign**

10 The charge, polarity, or sign of a compound refers to whether or not a compound has lost one or more electrons (positive charge, polarity, or sign) or gained one or more electrons (negative charge, polarity, or sign).

**Cell Targeting Signals**

15 Cell targeting signal (or abbreviated as the Signal) is defined in this specification as a molecule that modifies a biologically active compounds such as drug or nucleic acid and can direct it to a cell location (such as tissue) or location in a cell (such as the nucleus) either in culture or in a whole organism. By modifying the cellular or tissue location of the foreign gene, the function of the biologically active compound can be enhanced.

20 The cell targeting signal can be a protein, peptide, lipid, steroid, sugar, carbohydrate, (non-expresssing) polynucleic acid or synthetic compound. The cell targeting signal enhances cellular binding to receptors, cytoplasmic transport to the nucleus and nuclear entry or release from endosomes or other intracellular vesicles.

Nuclear localizing signals enhance the targeting of the pharmaceutical into proximity  
25 of the nucleus and/or its entry into the nucleus. Such nuclear transport signals can be a protein or a peptide such as the SV40 large T ag NLS or the nucleoplasmin NLS. These nuclear localizing signals interact with a variety of nuclear transport factors such as the NLS receptor (karyopherin alpha) which then interacts with karyopherin beta. The nuclear transport proteins themselves could also function as NLS's since they are targeted to the  
30 nuclear pore and nucleus. For example, karyopherin beta itself could target the DNA to the nuclear pore complex. Several peptides have been derived from the SV40 T antigen. These include a short NLS SEQ ID NO: 2 (H-CGYGPKKKRKVGG-OH) or long NLS's SEQ ID NOs: 3 & 4 (H-CKKKSSSDDEATADSQHSTPPKKKKRKVEDPKDFPSELLS-OH and H-CKKKWDDEATADSQHSTPPKKKKRKVEDPKDFPSELLS-OH). Other NLS peptides have

been derived from M9 protein SEQ ID NO: 5  
 (CYNDFGNYNQSSNFGPMKQGNFGGRSSGPY), E1A SEQ ID NO: 6 (H-CKRGPKRPRP-OH), nucleoplasmin SEQ ID NO: 7 (H-CKKAVKRPAATKKAGQAKKKKL-OH), and c-myc SEQ ID NO: 8 (H-CKKKGPAAKRVKLD-OH).

Signals that enhance release from intracellular compartments (releasing signals) can cause DNA release from intracellular compartments such as endosomes (early and late), lysosomes, phagosomes, vesicle, endoplasmic reticulum, golgi apparatus, trans golgi network (TGN), and sarcoplasmic reticulum. Release includes movement out of an intracellular compartment into cytoplasm or into an organelle such as the nucleus. Releasing signals include chemicals such as chloroquine, bafilomycin or Brefeldin A1 and the ER-retaining signal (KDEL sequence), viral components such as influenza virus hemagglutinin subunit HA-2 peptides and other types of amphipathic peptides.

Cellular receptor signals are any signal that enhances the association of the biologically active compound with a cell. This can be accomplished by either increasing the binding of the compound to the cell surface and/or its association with an intracellular compartment, for example: ligands that enhance endocytosis by enhancing binding the cell surface. This includes agents that target to the asialoglycoprotein receptor by using asialoglycoproteins or galactose residues. Other proteins such as insulin, EGF, or transferrin can be used for targeting. Peptides that include the RGD sequence can be used to target many cells. Chemical groups that react with thiol, sulfhydryl, or disulfide groups on cells can also be used to target many types of cells. Folate and other vitamins can also be used for targeting. Other targeting groups include molecules that interact with membranes such as lipids, fatty acids, cholesterol, dansyl compounds, and amphotericin derivatives. In addition viral proteins could be used to bind cells.

### **Interaction Modifiers**

An interaction modifier changes the way that a molecule interacts with itself or other molecules, relative to molecule containing no interaction modifier. The result of this modification is that self-interactions or interactions with other molecules are either increased or decreased. For example cell targeting signals are interaction modifiers that change the interaction between a molecule and a cell or cellular component. Polyethylene glycol is an interaction modifier that decreases interactions between molecules and themselves and with other molecules.

### Reporter or Marker Molecules

Reporter or marker molecules are compounds that can be easily detected. Typically they are fluorescent compounds such as fluorescein, rhodamine, Texas red, cy 5, cy 3 or dansyl compounds. They can be molecules that can be detected by infrared, ultraviolet or visible spectroscopy or by antibody interactions or by electron spin resonance. Biotin is another reporter molecule that can be detected by labeled avidin. Biotin could also be used to attach targeting groups.

### Linkages

An attachment that provides a covalent bond or spacer between two other groups (chemical moieties). The linkage may be electronically neutral, or may bear a positive or negative charge. The chemical moieties can be hydrophilic or hydrophobic. Preferred spacer groups include, but are not limited to C1-C12 alkyl, C1-C12 alkenyl, C1-C12 alkynyl, C6-C18 aralkyl, C6-C18 aralkenyl, C6-C18 aralkynyl, ester, ether, ketone, alcohol, polyol, amide, amine, polyglycol, polyether, polyamine, thiol, thio ether, thioester, phosphorous containing, and heterocyclic.

### Bifunctional

Bifunctional molecules, commonly referred to as crosslinkers, are used to connect two molecules together, i.e. form a linkage between two molecules. Bifunctional molecules can contain homo or heterobifunctionality.

### Crosslinking

Crosslinking refers to the chemical attachment of two or more molecules with a bifunctional reagent. A bifunctional reagent is a molecule with two reactive ends. The reactive ends can be identical as in a homobifunctional molecule, or different as in a heterobifunctional molecule.

### Amphiphilic and Amphipathic Compounds

Amphipathic, or amphiphilic, compounds have both hydrophilic (water-soluble) and hydrophobic (water-insoluble) parts. Hydrophilic groups indicate in qualitative terms that the chemical moiety is water-preferring. Typically, such chemical groups are water soluble, and are hydrogen bond donors or acceptors with water. Examples of hydrophilic groups include

compounds with the following chemical moieties; carbohydrates, polyoxyethylene, peptides, oligonucleotides and groups containing amines, amides, alkoxy amides, carboxylic acids, sulfurs, or hydroxyls. Hydrophobic groups indicate in qualitative terms that the chemical moiety is water-avoiding. Typically, such chemical groups are not water soluble, and tend not to hydrogen bonds. Hydrocarbons are hydrophobic groups.

### Detergent

Detergents or surfactants are water-soluble molecules containing a hydrophobic portion (tail) and a hydrophilic portion (head), which upon addition to water decrease water's surface tension. The hydrophobic portion can be alkyl, alkenyl, alkynyl or aromatic. The hydrophilic portion can be charged with either net positive (cationic detergents), negative (anionic detergents), uncharged (nonionic detergents), or charge neutral (zwitterionic detergent). Examples of anionic detergents are sodium dodecyl sulfate, glycolic acid ethoxylate(4 units) 4-*tert*-butylphenylether, palmitic acid, and oleic acid. Examples of cationic detergents are cetyltrimethylammonium bromide and oleylamine. Examples of nonionic detergents include, laurylmaltoside, Triton X-100, and Tween. Examples of zwitterionic detergents include 3-[(3-cholamidopropyl)dimethylammonio]1-propane-sulfonate (CHAPS), and N-tetradecyl-N,N-dimethyl-3-ammoniu-1-propanesulfonate.

### Surface Tension

The surface tension of a liquid is the force acting over the surface of the liquid per unit length of surface that is perpendicular to the force that is acting of the surface. Surface charge has the units force per length, *e.g.* Newtons/meter.

### Membrane Active Compound

Membrane active agents or compounds are compounds (typically a polymer, peptide or protein) that are able alter the membrane structure. This change in structure can be shown by the compound inducing one or more of the following effects upon a membrane: an alteration that allows small molecule permeability, pore formation in the membrane, a fusion and/or fission of membranes, an alteration that allows large molecule permeability, or a dissolving of the membrane. This alteration can be functionally defined by the compound's activity in at least one the following assays: red blood cell lysis (hemolysis), liposome leakage, liposome fusion, cell fusion, cell lysis and endosomal release. An example of a membrane active agent in our examples is the peptide melittin, whose membrane activity is

demonstrated by its ability to release heme from red blood cells (hemolysis). In addition, dimethylmaleamic-modified mellitin (DM-Mel) reverts to melittin in the acidic environment of the endosome causes endosomal release as seen by the diffuse staining of fluorescein-labeled dextran in our endosomal release assay.

5 More specifically membrane active compounds allow for the transport of molecules with molecular weight greater than 50 atomic mass units to cross a membrane. This transport may be accomplished by either the total loss of membrane structure, the formation of holes (or pores) in the membrane structure, or the assisted transport of compound through the membrane. In addition, transport between liposomes, or cell membranes, may be  
10 accomplished by the fusion of the two membranes and thereby the mixing of the contents of the two membranes.

### **Membrane active peptides.**

Membrane active peptides are peptides that have membrane activity. There are many  
15 naturally occurring membrane active peptides such as cecropin (insects), magainin, CPF 1, PGLa, Bombinin BLP-1 (all three from amphibians), melittin (bees), seminalplasmin (bovine), indolicidin, battenecin (both from bovine neutrophils), tachyplesin 1 (crabs), protegrin (porcine leukocytes), and defensins (from human, rabbit, bovine, fungi, and plants). Gramicidin A and gramicidin S (bacillus brevis), the lantibiotics such as nisin (lactococcus  
20 lactis), androctonin (scorpion), cardiotoxin I (cobra), caerin (frog litoria splendida), dermaseptin (frog). Viral peptides have also been shown to have membrane activity, examples include hemagglutinin subunit HA-2 (influenza virus), E1 (Semliki forest virus), F1 (Sendai and measles viruses), gp41 (HIV), gp32 (SIV), and vp1 (Rhino, polio, and coxsackie viruses). In addition synthetic peptides have also been shown to have membrane activity.  
25 Synthetic peptides that are rich in leucines and lysines (KL or KL<sub>n</sub> motif) have been shown to have membrane activity. In particular, the peptide SEQ ID NO: 9 H<sub>2</sub>N-KLLKLLKLWLKLLKLLKLL-CO<sub>2</sub>, termed KL<sub>3</sub>, is membrane active.

### **Polymers**

30 A polymer is a molecule built up by repetitive bonding together of smaller units called monomers. In this application the term polymer includes both oligomers which have two to about 80 monomers and polymers having more than 80 monomers. The polymer can be linear, branched network, star, comb, or ladder types of polymer. The polymer can be a

homopolymer in which a single monomer is used or can be copolymer in which two or more monomers are used. Types of copolymers include alternating, random, block and graft.

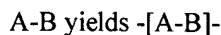
The main chain of a polymer is composed of the atoms whose bonds are required for propagation of polymer length. For example in poly-L-lysine, the carbonyl carbon,  $\alpha$ -carbon, and  $\alpha$ -amine groups are required for the length of the polymer and are therefore main chain atoms. The side chain of a polymer is composed of the atoms whose bonds are not required for propagation of polymer length. For example in poly-L-lysine, the  $\beta$ ,  $\gamma$ ,  $\delta$ , and  $\epsilon$ -carbons, and  $\epsilon$ -nitrogen are not required for the propagation of the polymer and are therefore side chain atoms.

To those skilled in the art of polymerization, there are several categories of polymerization processes that can be utilized in the described process. The polymerization can be chain or step. This classification description is more often used than the previous terminology of addition and condensation polymer. "Most step-reaction polymerizations are condensation processes and most chain-reaction polymerizations are addition processes" (M. P. Stevens Polymer Chemistry: An Introduction New York Oxford University Press 1990). Template polymerization can be used to form polymers from daughter polymers.

#### Step Polymerization:

In step polymerization, the polymerization occurs in a stepwise fashion. Polymer growth occurs by reaction between monomers, oligomers and polymers. No initiator is needed since there is the same reaction throughout and there is no termination step so that the end groups are still reactive. The polymerization rate decreases as the functional groups are consumed.

Typically, step polymerization is done either of two different ways. One way, the monomer has both reactive functional groups (A and B) in the same molecule so that



Or the other approach is to have two difunctional monomers.



Generally, these reactions can involve acylation or alkylation. Acylation is defined as the introduction of an acyl group ( $-COR$ ) onto a molecule. Alkylation is defined as the introduction of an alkyl group onto a molecule.